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Metagenomic and metatranscriptomic analysis of the microbial community in Swiss-type Maasdam cheese during ripening

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ABSTRACT

In Swiss-type cheeses, characteristic nut-like and sweet flavor develops during the cheese ripening due to the metabolic activities of cheese microbiota. Temperature changes during warm and cold room ripening, and duration of ripening can significantly change the gene expression of the cheese microbiota, which can affect the flavor formation. In this study, a metagenomic and metatranscriptomic analysis of Swiss-type Maasdam cheese was performed on samples obtained during ripening in the warm and cold rooms. We reconstructed four different bacterial genomes (*Lactococcus lactis*, *Lactobacillus rhamnosus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii* subsp. *shermanii* strain JS) from the Maasdam cheese to near completeness. Based on the DNA and RNA mean coverage, *L. lactis* strongly dominated (~80–90%) within the cheese microbial community. Genome annotation showed the potential for the presence of several flavor forming pathways in these species, such as production of methanethiol, free fatty acids, acetoin, diacetyl, acetate, ethanol, and propionate. Using the metatranscriptomic data, we showed that, with the exception of *L. lactis*, the central metabolism of the microbiota was downregulated during cold room ripening suggesting that fewer flavor compounds such as acetoin and propionate were produced. In contrast, *L. lactis* genes related to the central metabolism, including the vitamin biosynthesis and homolactic fermentation, were upregulated during cold room ripening.

1. Introduction

The microbiota of cheese is one of the most important factors in cheese ripening and flavor forming. Cheese flavor development is a dynamic and complex biochemical process, in which the environmental conditions, including cooking temperature, ripening time, and temperature, affect the microbial composition and metabolic activity (Lawlor et al., 2003). The ripening temperature and the ripening time of Swiss-type cheese can vary depending on the cheese producer. Generally, Swiss-type cheeses are ripened for 30 to 60 days in a warm room (20–24 °C) and then transferred to a cold room (4–11 °C) for another 30 to 60 days (Guggisberg et al., 2015). Transferring the cheese from a warm room to a cold room is critical for eye formation control in Swiss-type cheeses. The eye formation requires sufficient time in a warm room, and after enough holes are formed, cheese needs to be transferred to a cold room to avoid excessive gas production (Daly et al., 2010;

Guggisberg et al., 2015). Cheese flavor has an enormous impact on consumer enjoyment and preferences (Liggett et al., 2008). Therefore, the development of the desired flavor is critical for the cheese industry. Several studies have been performed to better understand the flavor formation in cheeses (Murtaza et al., 2014; Smit et al., 2005). Bacterial species that are used for cheese production vary depending on the type of the cheese. In Swiss-type cheeses, *Lactobacillus* species and *Streptococcus thermophilus* are the typical starter cultures (Jenkins et al., 2002). The starter cultures are mainly responsible for fast acidification by fermentation of cheese sugars, and peptide and amino acid production from casein (Johnson, 2013). In addition to lactobacilli and *S. thermophilus*, *Propionibacterium freudenreichii* is a key species for the Swiss-type cheese production, since its growth is associated with propionate (typical flavor) and CO₂ production (eye formation) during fermentation of lactate produced by the starter cultures (Ojala et al., 2017). Adjunct mesophilic *Lactobacillus* can also be added to Swiss-type cheeses, since

Abbreviations: TCA, tricarboxylic acid cycle; Padj, adjusted *p*-value; MIMAG, Minimum information about a metagenome-assembled genome

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they have positive effects on flavor formation (Bouton et al., 2009; Kocaoglu-Vurma et al., 2008). As a result of microbial activities, the balance of produced free fatty acids, peptides, amino acids and carbonyl group compounds provides the characteristic nut-like and sweet flavors to Swiss-type cheeses (Beuvier et al., 1997).

Due to the development of next-generation sequencing techniques, application of metagenomic and metatranscriptomic methods has become more common in food-related research (Kergourlay et al., 2015). Recently, metagenomic or metatranscriptomic analyses of Italian cheese (De Filippis et al., 2016), French cheese (Ceugniet et al., 2017; Dugat-Bony et al., 2015), Mexican cheese (Escobar-Zepeda et al., 2016) and Belgian cheese (Deltcenserie et al., 2014) have been performed, producing new knowledge regarding diversity and metabolic features of microbial species in cheeses. To our knowledge, Swiss-type cheese has not been studied using metagenomics and metatranscriptomic analyses prior to our work.

Previously, from the same cheese samples that were used in this study, the transcriptomic analysis of *P. freudenreichii* subsp. *shermanii* JS in Swiss-type Maasdam cheese has been done (Ojala et al., 2017). That study only focused on *P. freudenreichii* and reported that the genome of *P. freudenreichii* possesses genes that play a role in production of flavor compounds, such as acetoin, acetate and propionate. In addition, the study showed that central carbon metabolism genes and propionate production genes of *P. freudenreichii* are downregulated during cold room ripening (Ojala et al., 2017). The analysis of the whole cheese microbiota and gene expression profiles of other than *P. freudenreichii* species provides additional knowledge about Swiss-type cheeses ripening and flavor formation. In this study, the whole metagenome and metatranscriptome of the semi-hard Swiss-type Maasdam cheese were generated to characterize the cheese microbiota, reconstruct their genomes, and compare their gene expression profiles during warm and cold room ripening.

2. Materials and methods

2.1. Cheese sampling

For this study, semi-hard Maasdam-type cheeses (moisture 51.1%, fat 15%, and salt 1.5%) were manufactured in a cheese plant with good manufacturing practice. Cheeses were cooked from pasteurized milk in closed vats. Standard mesophilic Gouda cheese process (Fox and McSweeney, 2004) was applied using mesophilic *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (optimum growth temperature is 30–36 °C (Chen et al., 2015)) as primary acidifiers. Mesophilic *Propionibacterium freudenreichii* subsp. *shermanii* JS and *Lactobacillus rhamnosus*, as well as thermophilic *Lactobacillus helveticus* were used as adjunct cultures. After cooking process, molding, pressing, and brining were applied to cheeses. Three of the 13 kg cooked cheeses named A, B, and C were cut into four parts and wrapped with ripening foil for the ripening step. First 30 days, cheeses were ripened in the warm room at +20 °C. After, those cheeses were transferred into the +5 °C cold room and ripening was continued for another 30 days. The first triplet samples were collected from the warm room on day 12 of ripening (samples A1, B1, and C1). The second triplet samples (A3, B3, C3) were collected during storage in the cold room on day 37 (after seven days in cold room) (Fig. 1).

2.2. DNA extraction, DNA sequencing, and metagenomics analysis

Ten grams of finely grated cheese was blended with 90 g of 2% tri-sodium citrate, mixed for 2.5 min with Stomacher blender in filter pouch. Cells were collected from 1 ml of suspension by centrifuging for 2 min at 14,000 ×g, and further utilized for DNA extraction. DNA extraction was performed with Wizard® genomic DNA Purification Kit (Promega) with modifications in lysis step as described earlier (Ahluwalia and Tynkynen, 2009) applying 50 mM EDTA, 10 mg/ml Lysozyme

(Amresco), 100 U/ml mutanolysin (Sigma-Aldrich) and incubating the cells at 37 °C for 1 h for lysis. DNA concentration and purity were analyzed by Nanodrop ND –1000 spectrophotometer (NanoDrop Technologies, Inc.).

Prepared multiplexed libraries using Nextera XT approach (Illumina) were sequenced by using the Illumina MiSeq in paired-end manner (326 + 286 bp paired-end read chemistry) in eleven different sequencing runs with other multiplexed amplicon libraries from other projects. Due to rather well-known issue of index hopping or switching on Illumina platform (Illumina Inc., 2017) we removed all those read pairs that contained index hopped reads, which were from other multiplexed amplicon libraries from other projects.

After adapter trimming using Cutadapt v1.13 (Martin, 2011), Trimmomatic v0.36 (Bolger et al., 2014) was used for quality filtering. Reads that contained < 100 base pairs and bases that had Phred quality score < 25 were filtered out (Supplemental file 1). After the data classification and taxonomic analysis (Supplemental file 1), metaSPAdes v3.10.1 (Nurk et al., 2017) was used for *de novo* assembly. Assembled contigs were grouped into bins by using MYCC with minimum contig length 1000 bp option (Lin and Liao, 2016) to reconstruct representing genomes (Supplemental file 2). CheckM v1.0.7 (Parks et al., 2015) was used for quality analysis of genome bins. Lineage-specific workflow was applied for each bin. DNA reads were mapped against bins of contigs by using BWA v0.7.15 (Li and Durbin, 2009) to estimate coverage of bins. The mean coverage of mapped reads was calculated by using Qualimap v2.2.1 (Garcia-Alcalde et al., 2012).

2.3. Phylogenetic analysis of bins

Phylogenetic relationship between bins and close strains was investigated by constructing maximum likelihood phylogenetic tree. For each bin, the genomes of the closest strains and outgroup strains were downloaded from NCBI database (July 2017). We identified orthologous proteins for each bin and downloaded genomes using Proteinortho v5.16 (Lechner et al., 2011) with searching options of e-value 1e-05. Identified orthologous proteins (697 proteins for *Lc. lactis*, 950 proteins for *Lb. helveticus*, 1650 proteins for *Lb. rhamnosus* and 1121 proteins for *P. freudenreichii*) for each strain were aligned using MUSCLE v3.8.31 (Edgar, 2004). The alignment set was concatenated for each strain, and Gblocks v0.91b (Castresana, 2000) was used to remove poorly aligned positions. FastTree v2.1.10 (Price et al., 2010) was used for phylogenetic tree computation. For visualization of the trees, SplitsTree v4.14.4 (Huson and Bryant, 2006) was used.

Every bin was mapped against the genomes of close related species using ABACAS v1.3.1 (Assefa et al., 2009) to align, order, and orientate the contigs. These alignments were visualized using BRIG software v0.95 (Alikhan et al., 2011).

2.4. Genome bins annotation and pathway reconstruction

Created bins were annotated by using PROKKA pipeline v1.12 (Seemann, 2014). In addition, PANNZER (Koskinen et al., 2015), Blast2GO v4.1.8 (Conesa et al., 2005) and KAAS v2 (Moriya et al., 2007) were also used for EC number and GO term annotation (Supplemental file 3). Based on annotations, pathway reconstruction was done using Pathway Tools v20.0 (Karp et al., 2016).

2.5. RNA extraction, RNA sequencing, and Metatranscriptome analysis

Cheese microbes for RNA extraction were isolated as described by Ojala et al. (2017). Briefly, 10 g of finely grated cheese was blended with 2% tri-sodium citrate in a Stomacher filter pouch and mixed with Stomacher blender. The whole liquid part was centrifuged, liquid and fat on the surface were carefully removed and the pellet was suspended in RNA Protect Bacteria Reagent (Qiagen). The suspension was transferred to a clean tube, mixed and incubated for 5 min at room

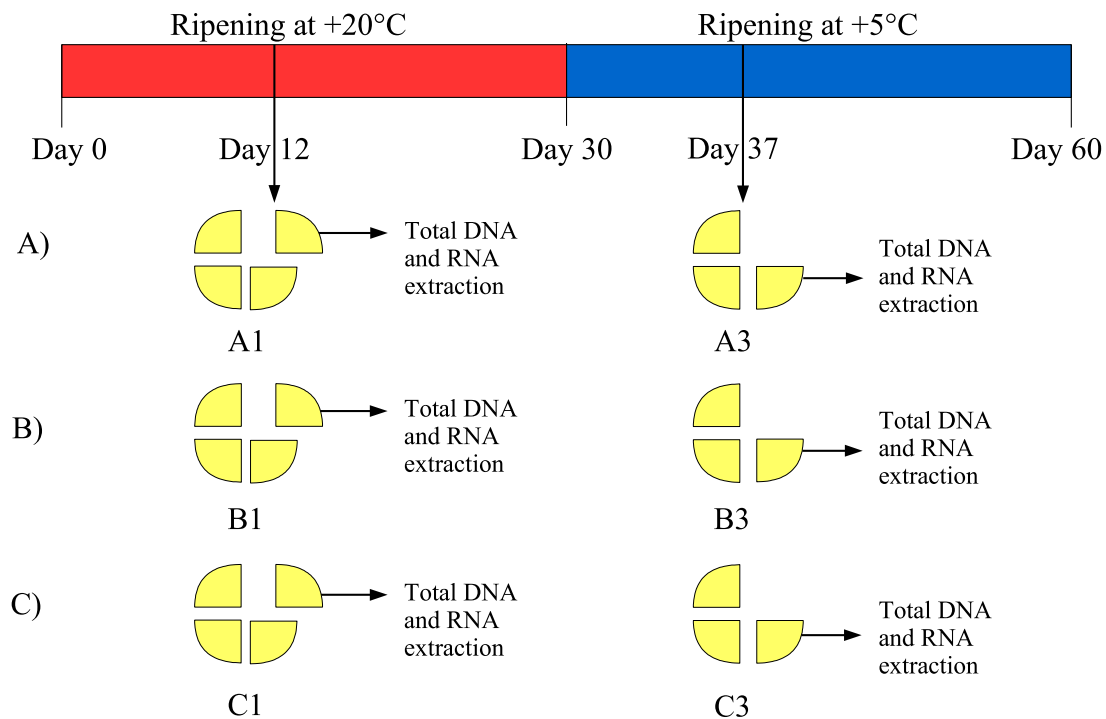


Fig. 1. Cheese sampling. Cooked semi-hard Maasdam-type cheeses were ripened 30 days in the warm room and 30 days in the cold room. Samples were collected on the day 12 (time point 1, samples A1, B1, and C1), and day 37 (time point 2, samples A3, B3, and C3).

temperature, then centrifuged, followed by liquid and fat removal. The cell pellet was further used for RNA extraction as described by Koskenniemi et al. (2011). Cells were lysed with 10 mg/ml of lysozyme (Amresco), 3 mg/ml proteinase K (Sigma-Aldrich), and 100 U/ml of mutanolysin (Sigma-Aldrich) at 37 °C for 30 min. Next, 1 ml of pre-heated (65 °C) TRIzol reagent (Invitrogen) was added to the suspension and vortexed for 3 min. The suspension was incubated at room temperature for 5 min and homogenized in a MagNA Lyser instrument (Roche Applied Science) with < 106-μm glass beads (Sigma-Aldrich) for four 30 s cycles at 6000 rpm. Cell debris was removed by centrifugation, and the lysate extracted with 200 μl of chloroform by vortexing for 15 s. The phases were separated by centrifugation after 3 min incubation at room temperature. 500 μl of 80% ethanol was added to the aqueous phase for RNeasy Mini kit (Qiagen) total RNA purification. The concentration and purity of RNA samples were determined by NanoDrop ND – 1000 spectrophotometer and denaturing agarose gel electrophoresis.

RIBOZero kit (Epicentre) was used for removing rRNA by following manufacturer's instructions. RNA fractions were amplified as cDNA and libraries were constructed by using Ovation RNA-seq System V2 (NuGEN Technologies Inc., San Carlos, CA). Constructed libraries were sequenced by using the SOLiD 5500XL (Life technologies) sequencer with single-end sequencing method (75 bp single-read chemistry) using non-strand-specific protocol.

Quality filtering and adapter trimming of the reads were done by using Cutadapt v1.13 (Martin, 2011). Reads that contained < 30 base pairs and low-quality ends (which have < 25 Phred quality score) were filtered out. rRNA sequences were filtered out using SortMeRNA v2.1 (Kopylova et al., 2012). Filtered reads were mapped onto the bins using STAR aligner v2.5.2b (Dobin et al., 2013). HTSeq v0.9.0 (Anders et al., 2015) was used for counting the reads that mapped to each gene. Normalization of the counts and differential gene expression analysis was done using DESeq2 v1.18.1 (Love et al., 2014) between two conditions, warm and cold room ripening. Warm room ripening gene expressions were used as a reference. Correction of multiple testing was done using the Benjamini-Hochberg procedure (Benjamini and

Hochberg, 1995), which provides false discovery rate. Genes with Benjamini-Hochberg adjusted p -value (p_{adj}) ≤ 0.10 and $|\log_2 \text{Fold Change}| \geq 1$ were considered as significantly differentially expressed. Genes that had three or more samples with DESeq2 normalized counts less or equal to four were assigned as genes with low expression. Functional enrichment analysis of the differentially expressed genes was done by using Blast2GO v4.1.8 (Conesa et al., 2005) and Pathway Tools v20.0 (Karp et al., 2016). For both software, p -value threshold of 0.05 and Fisher's exact test was used.

3. Results

3.1. Genome reconstruction from cheese metagenome

A total length of 10.5 Mbp contigs with minimum 1000 bp were created by metagenomic assembling of total DNA reads (Table S6a in Supplemental file 2). During genome reconstruction, contigs were grouped into four taxonomically different genome bins. *Lactococcus lactis* species, *Lactobacillus rhamnosus* species, *Lactobacillus helveticus* species, and *Propionibacterium* genus lineage-specific marker sets were determined for each bin by CheckM (Table 1). According to quality assessment from CheckM results, the genome completeness of all the bins was higher than 97% (Table 1). Mean coverage results (Fig. 2) showed that, the mean DNA read coverage for *Lc. lactis* bin was dramatically higher compared to other bins. Between warm and cold ripening, mean coverage of *Propionibacterium* bin increased significantly (p -value < 0.01, Welch's t -test (Welch, 1938)).

3.2. Phylogenetic analysis of genome bins

For phylogenetic analysis four trees were created. For *Lc. lactis* bin tree construction, 19 genomes of *Lc. lactis* subsp. *lactis* strains and 12 *Lc. lactis* subsp. *cremoris* strains were used with one genome of *Streptococcus thermophilus* as an outgroup. For *Lb. helveticus* bin, 10 genomes of *Lb. helveticus* were used with the genome of *Lactobacillus acidophilus* as an outgroup. Created trees demonstrated that the closest strains to the

Table 1

Created four bins from assembly contigs using MYCC binning and CheckM quality results.

Genome Bins	Marker lineage for CheckM ^a	Completeness (%)	Contamination (%) ^b
Bin 1	<i>Lc. lactis</i>	99.62	2.3
Bin 2	<i>Lb. helveticus</i>	98.38	0
Bin 3	<i>Propionibacterium</i>	98.14	0.88
Bin 4	<i>Lb. rhamnosus</i>	97.42	0.16

^a Marker lineage determined automatically from CheckM software, by placing the genome bin within a reference genome tree.

^b CheckM contamination estimation of bins determined by presence of multi-copy marker genes within genome bins.

genomic bins were *Lc. lactis* subsp. *lactis* strain IL1403 (Bolotin et al., 2001) and *Lb. helveticus* strain CNRZ32 (Broadbent et al., 2013) (Fig. S5 and S6 in Supplemental file 4). For *Lactobacillus rhamnosus* and *Propionibacterium* bins, nine *Lb. rhamnosus* strains, one *Lb. casei* genome, four *P. freudenreichii* genomes, and one *Cutibacterium acnes* genome, respectively, were used for phylogenetic tree construction. *Lb. rhamnosus* strain LC705 (Kankainen et al., 2009) and *P. freudenreichii* subsp. *shermanii* strain JS (Ojala et al., 2017) were the closest strains for *Lb. rhamnosus* and *Propionibacterium* bins, respectively (Fig. S7 and S8 in Supplemental file 4).

Ring images were created by mapping bins to the closest strains. The visualized alignments (Fig. 3) showed that four reconstructed genome bins were nearly complete.

3.3. Cheese ripening and flavor formation metabolic pathways of cheese microbiota

Annotation and pathway reconstruction allowed us to detect three most important metabolic pathways related to cheese ripening; fermentation, lipolysis, and proteolysis, which are responsible for texture and flavor formation (Smit et al., 2005).

3.3.1. Proteolysis and amino acid degradation

All reconstructed genome bins except *P. freudenreichii* had genes required for proteinase activity for casein. Several genes for peptide

transportation and peptidases were found in all lactic acid bacteria strains (Fig. 4). *P. freudenreichii* had genes for a putative peptide transport permease protein and a putative oligopeptide transport protein. Furthermore, some peptidase genes were annotated in *P. freudenreichii*, which encode PepO, PepC, PepE, methionine aminopeptidase and proteasome endopeptidase complex.

In addition, genes for transaminases were found in all species. In total 12 proteinogenic amino acids predicted to be degraded by Maasdam cheese microbiota (Supplemental file 5). Catabolic pathways for aromatic amino acids were not found in the reconstructed bins. Valine degradation was the only branched-chain amino acid degradation pathway for the Maasdam cheese microbiota. *Lc. lactis* was predicted to degrade valine by 'valine degradation II' pathway with formation of isobutanol, while *P. freudenreichii* subsp. *shermanii* strain JS had 'valine degradation I' pathway, which produces 2-methylpropanal. Required pathway genes for sulfur containing amino acids (methionine and cysteine) degradation were found in the genomes of *Lc. lactis*, *Lb. rhamnosus*, and *Lb. helveticus*.

3.3.2. Lipolysis and metabolism of fatty acids

Annotation results and pathway analysis showed that *Lc. lactis*, *Lb. rhamnosus* and *P. freudenreichii* subsp. *shermanii* strain JS have genes for lipases and esterases to release fatty acids from triglycerides. In addition, all four bacteria found in Maasdam cheese possessed genes for fatty acid biosynthesis from acetyl-CoA. Palmitate, stearate, palmitoleate and vaccenate were predicted to be synthesized *de novo* by Maasdam cheese microbiota.

3.3.3. Fermentation

Annotations of genomic bins suggested that only *Lc. lactis* and *Lb. rhamnosus* use phosphotransferase system for lactose transportation in Maasdam cheese microbiota. Lactose permease (*lacS*) was only found in *Lc. lactis* and *Lb. helveticus*; however, the presence of the gene *lacZ* in *P. freudenreichii* subsp. *shermanii* strain JS and *lacM* and *lacL* in *Lb. rhamnosus* indicated that these two strains might also degrade lactose to galactose. In addition, all species were equipped with genes, which are required for the Leloir pathway (Fig. 5).

The intermediate products of lactose degradation pathways were predicted to be catabolized through glycolysis to produce pyruvate, which is the precursor of several flavor compounds in cheese, such as

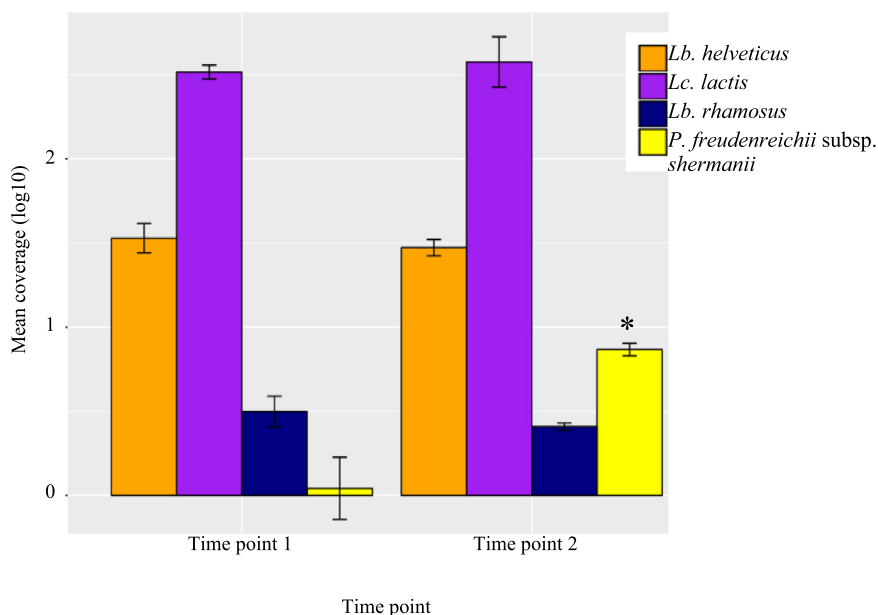


Fig. 2. Mean DNA read coverage of every genomic bin for two different time points. Error bars represent standard deviation. For Y-axis, log10 transformation of values was used for better visualization. *: *p*-value < 0.01 (Welch Two Sample *t*-test).

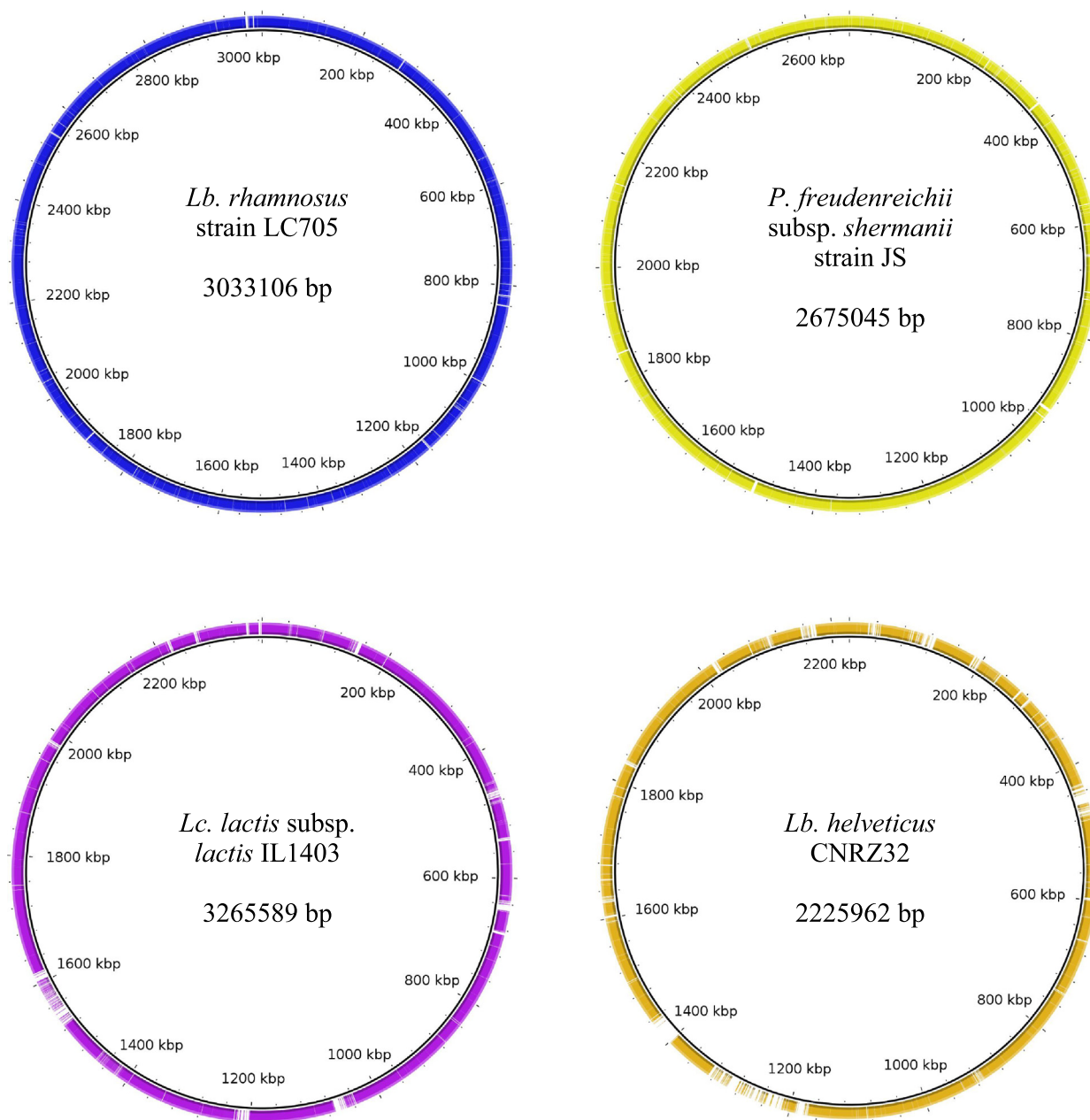


Fig. 3. Genome reconstruction from Maasdam cheese metagenome. Inner black ring represents the reference genome (names are shown in the center of rings) and outer ring is alignment of bins to references.

ethanol, lactate, acetate, diacetyl, acetoin, and butanediol (Fig. 6). All four species in Maasdam microbiota had genes responsible for producing L-lactate from pyruvate. However, we suggest that D-lactate cannot be produced by *P. freudenreichii* subsp. *shermanii* strain JS due to the lack of D-lactate dehydrogenase gene. R-acetoin and diacetyl, which are buttery flavor compounds (Martino et al., 2016), could potentially be produced by *Lc. lactis*, *Lb. rhamnosus*, and *P. freudenreichii* subsp. *shermanii* strain JS. In addition, the reduced form of R-acetoin, 2–3 butanediol, was predicted to be produced by *Lc. lactis* due to the presence of diacetyl reductase gene in the genome. In turn, *P. freudenreichii* had genes for conversion of lactate to pyruvate, and pyruvate to propionate by Wood-Werkman cycle and tricarboxylic acid (TCA) cycle with concomitant production of CO₂.

In addition to lactose, citrate, which is found in milk in soluble phase (McGann et al., 1983), could be degraded by Maasdam cheese

microbiota. Citrate fermentation produces two main compounds for cheese; acetate and pyruvate. Pyruvate produced from citrate is primarily converted to acetoin and diacetyl that enhance flavor (Martino et al., 2016). Our annotation implied that *Lb. rhamnosus* and *Lb. helveticus* have *citD*, *citF*, and *citE* genes encoded citrate lyase. However, oxaloacetate decarboxylase gene was only found in *Lb. rhamnosus*. Therefore, *Lb. rhamnosus* is presumably the only species potentially capable to degrade citrate to pyruvate.

3.4. Vitamin synthesis

The gene annotation revealed that several B group vitamins were predicted to be produced by Maasdam cheese microbiota. All four bacteria contained genes for production of folic acid (B₉), thiamine (B₁), and pyridoxine (B₆). Flavin biosynthesis genes were found only in

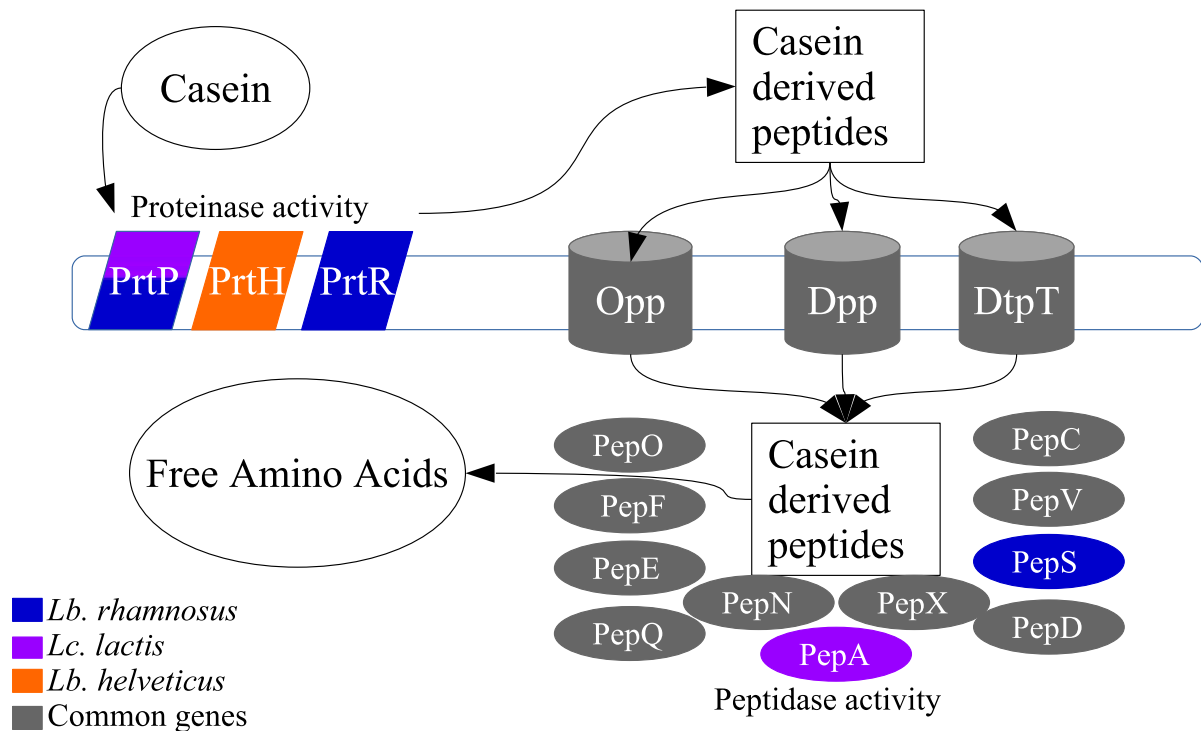


Fig. 4. Summary of proteinase and peptidase activities in Maasdam cheese. Gray colored genes are genes present in all three lactic acid bacteria species, species specific genes shown with specific colors. Casein is degraded to peptides by PrtP, PrtH, and PrtR membrane proteases. Peptides are transported into cell by oligopeptide (Opp), dipeptide (Dpp) and di/tripeptide (DtpT) transporters and hydrolyzed thereafter into amino acids by several peptidases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Lc. lactis and *P. freudenreichii* subsp. *shermanii* strain JS. In addition, cobalamin (B₁₂) biosynthesis genes were found only in *P. freudenreichii* subsp. *shermanii* strain JS genome.

3.5. Gene expression profiling and differential expression analysis

RNA-seq mapping to genome bins showed that similarly to the mean coverage of shotgun DNA sequencing, *Lc. lactis* bin dramatically dominated the cheese samples. Approximately ~90% and ~85% of RNA reads mapped uniquely to *Lc. lactis* bin at time point 1 (the warm room) and 2 (the cold room), respectively. We observed that between warm room and cold room ripening, the percentage of reads uniquely mapped to *Lc. lactis* bin slightly decreased, while for other bins it increased (Table 2).

Based on the normalized read counts from DESeq2, the number of low expressed genes in *Lb. helveticus* bin was high; 75% of annotated genes were found to be low expressed during cheese ripening. The number of expressed genes of other bins were high (Table 2).

Differential gene expression analysis between warm and cold room ripening revealed several upregulated and downregulated genes in Maasdam cheese microbiota. For all species, number of downregulated genes were slightly higher compared to number of upregulated genes during cold room ripening (Table 2, Supplemental file 6).

3.6. Differentially expressed genes and gene enrichment analysis

In gene enrichment analysis we determined several different GO terms and metabolic pathways that were enriched with significantly upregulated and downregulated genes. Enriched GO terms and pathways were mostly related to the fermentation, carbohydrate transport, carbohydrate metabolism, alternative sugar degradation, nucleotide biosynthesis and fatty acid biosynthesis (Table 3, Supplemental files 7 and 8).

4. Discussion

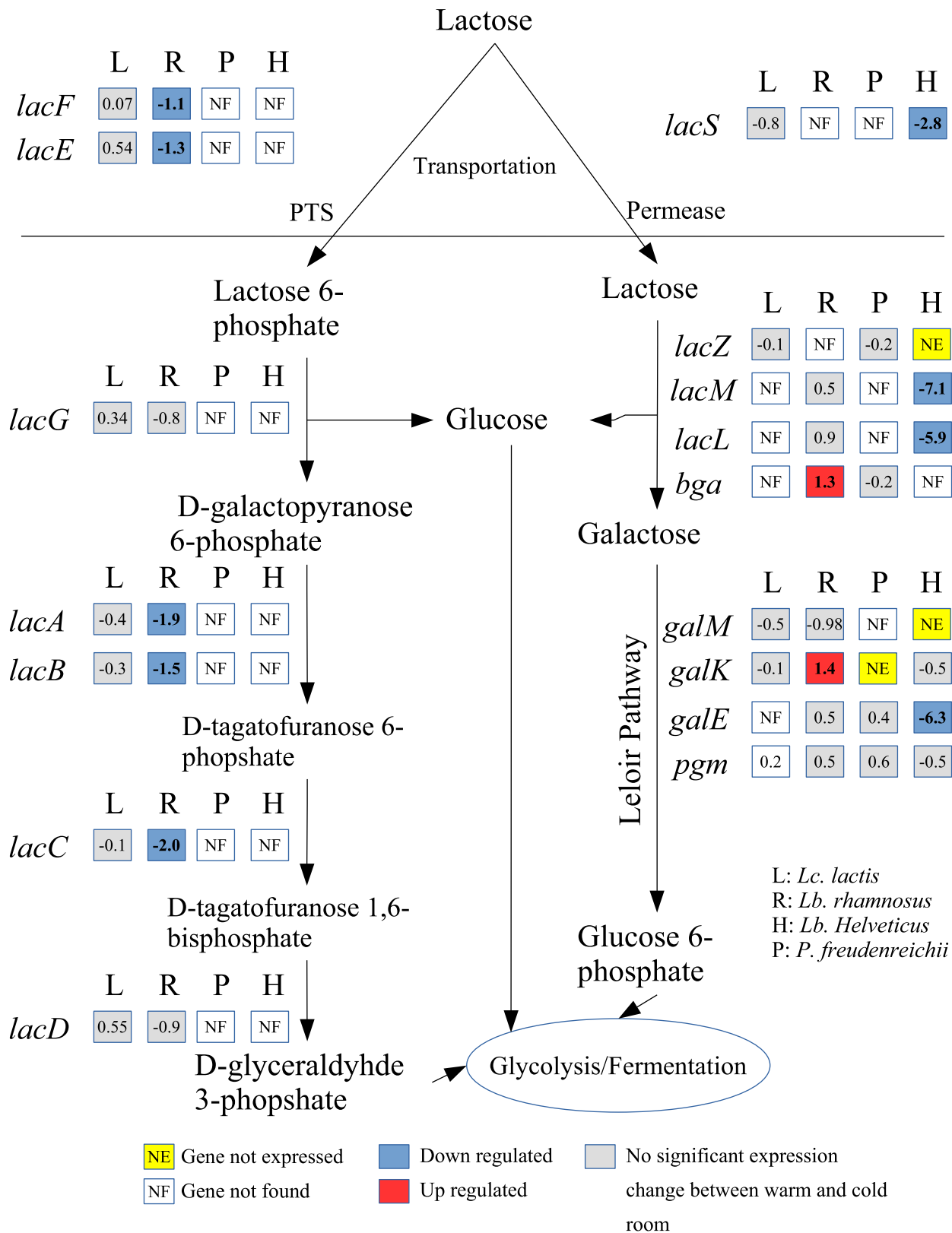
During the ripening process of Swiss-type cheeses there are two main stages of fermentation: lactose fermentation to lactate in the early stages performed by lactic acid bacteria, such as *Lc. lactis*, *Lb. rhamnosus*, and *Lb. helveticus*. Later on, lactate is fermented to propionate, CO₂ and acetate by *P. freudenreichii* (Jenkins et al., 2002; White et al., 2003).

In the beginning of this study, the cheese manufacturer indicated that a five strain mixture of *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lb. rhamnosus*, *Lb. helveticus*, and *P. freudenreichii* subsp. *shermanii* JS were used together in production of Swiss-type Maasdam cheese. However, with metagenomic analysis we were able to construct only one *Lc. lactis* genomic bin. It is possible that the two *Lc. lactis* genomes were merged during the assembly process. The metagenomic analysis allowed us to reconstruct four genome bins within the cheese microbiota. The previous study that used the data from the same cheese samples (Ojala et al., 2017) identified *P. freudenreichii* subsp. *shermanii* strain JS. The phylogenetic analysis showed that *Lc. lactis* subsp. *lactis* strain IL1403, *Lb. helveticus* strain CNRZ32, and *Lb. rhamnosus* LC705 were the closest strains to the identified genome bins of *Lc. lactis*, *Lb. helveticus*, and *Lb. rhamnosus*. Furthermore, we detected low abundance DNA reads that belonged to other than to the added starter cultures species, such as *Enterococcus faecium*, *Leuconostoc mesenteroides*, and *Streptococcus thermophilus* (Table S1–S4 in Supplemental file 1). Even though pasteurized milk was used for the cheese making, the milk was not sterile. Therefore, it is expected that we see other milk environmental bacteria in the cheese microbiota (Beuvier et al., 1997; Quigley et al., 2012). In addition, brining stage can also introduce additional bacterial species to cheese microbiota (Marino et al., 2017). Furthermore, the cheeses were made from one cooking batch, but three separate cheeses were molded, pressed, brined and ripened separately. It was shown that even in cheeses made from same milk, the use of

different production equipment can cause variation in cheese microbiota (Williams et al., 2002).

According to the standards of minimum information about a metagenome-assembled genome (MIMAG) (Bowers et al., 2017),

assembled genome bins were high-quality drafts. All bins complied with the standards of high-quality draft, which is > 90% complete with < 5% contamination. The contamination rate of bins shows the presence of multiple copies of the marker genes in the reconstructed bins (Parks



(caption on next page)

Fig. 5. Lactose catabolic and transport pathways and their differential expression during transition from the warm room to the cold room. Letters are representing bacterial strains, L: *Lc. lactis*; R: *Lb. rhamnosus*; P: *P. freudenreichii* subsp. *shermanii* strain JS and H: *Lb. helveticus*. Numbers in boxes indicate log2 fold change results between the warm and cold room. Genes that were not found by genome annotation were marked as 'NF'. Few or zero RNA read mapped genes were marked as 'NE'. Blue box and red box represent significant downregulation and upregulation during cold room ripening, respectively. The names of enzymes: *lacF*-Lactose-specific phosphotransferase enzyme IIA, *lacE*-Lactose-specific phosphotransferase enzyme IIBC, *lacS*-Lactose permease, *lacG*-6-phospho-beta-galactosidase, *lacA*-Galactose-6-phosphate isomerase subunit A, *lacB*-Galactose-6-phosphate isomerase subunit B, *lacC*-Tagatose-6-phosphate kinase, *lacD*-Tagatose 1,6-diphosphate aldolase, *lacZ*-Beta-galactosidase, *lacM*-Beta-galactosidase small subunit, *lacL*-Beta-galactosidase large subunit, *bga*-Beta-galactosidase, *galM*-Aldose 1-epimerase, *galK*-Galactokinase, *galE*-UDP-glucose 4-epimerase, *pgm*-Phosphoglucumutase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

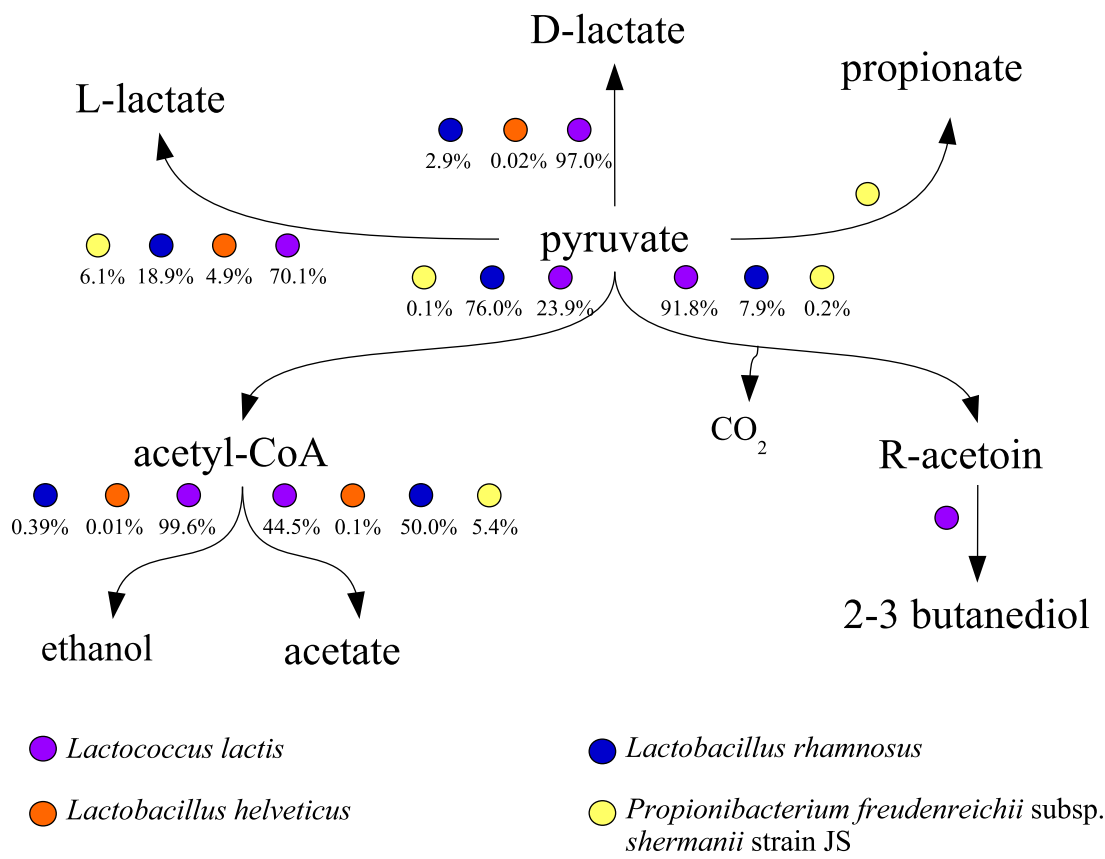


Fig. 6. Predicted pyruvate fermentation pathways in four strains of *Lc. lactis*, *Lb. helveticus*, *Lb. rhamnosus*, and *P. freudenreichii* subsp. *shermanii*. Colored circles are representing the found necessary genes for a metabolic pathway. Numbers under circles are showing contribution of species to each pathway by representing proportion of expressed pathway related genes amount in all samples (Calculated by average of normalized gene counts in each sample). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2015). In this study, contamination rate of the reconstructed bins was low (Table 1) with the maximum of ~2.5%, for *Lc. lactis* bin. This suggests that reconstructed *Lc. lactis* genome bin may contain small portion of other *Lactococcus* species, which were present in the cheese microbiota.

Even though completion levels of the reconstructed bins were high, alignment to the reference genomes showed that there were some missing parts for *Lc. lactis* and *Lb. helveticus* bins (Fig. 3). When we analyzed missing parts, we found that missing regions for both bins in comparison with their references included mostly mobile elements and

Table 2
The number of annotated protein-coding genes, expressed and low expressed genes at time point 1 (Tp1 warm room) and 2 (Tp2 cold room). In addition, number of upregulated and downregulated genes during cold room ripening are shown.

	#Protein coding genes annotated	#Low expressed genes + %	#Expressed genes + %	#Total Tp1 (warm room) mapped reads + % of mapped reads	#Total Tp2 (cold room) mapped reads + % of mapped reads	#Upregulated genes	#Downregulated genes
<i>Lc. lactis</i>	3018	267 (8.8%)	2751 (91.2%)	110,737,957 (90.27%)	105,946,893 (85.10%)	92	195
<i>Lb. rhamnosus</i>	2728	72 (2.6%)	2656 (97.4%)	8,844,465 (7.03%)	12,516,125 (10.16%)	158	181
<i>P. freudenreichii</i>	2248	795 (35.4%)	1453 (64.6%)	315,211 (0.27%)	2,553,051 (2.10%)	146	147
<i>Lb. helveticus</i>	2234	1684 (75.4%)	550 (24.6%)	2,416,388 (1.97%)	2,889,883 (2.34%)	12	53

Table 3

Cheese ripening related GO terms and pathway names that were enriched with upregulated and downregulated genes during cold room ripening.

Upregulated		Downregulated	
a) <i>Lc. lactis</i>			
GO term	p-Value	GO term	p-Value
Carbohydrate transmembrane transport	9.8E-05	Intramolecular lyase activity	5.0E-03
Vitamin biosynthetic process	1.3E-04	D-ribose metabolic process	5.0E-03
L-lactate dehydrogenase activity	1.0E-02	Pentose metabolic process	2.7E-02
Carbohydrate derivative metabolic process	3.9E-02	Regulation of primary metabolic process	3.0E-02
Pathway name	p-Value	Pathway name	p-Value
D-fructuronate degradation	7.5E-03	Ribose degradation	2.6E-03
D-Glucuronate degradation	1.2E-02	Sugars degradation	1.5E-02
Pyruvate fermentation to lactate	1.2E-02	Sugar nucleotides biosynthesis	1.7E-02
Vitamins biosynthesis	3.5E-02	Carbohydrates degradation	2.1E-02
b) <i>Lb. rhamnosus</i>			
GO term	p-Value	GO term	p-Value
Purine nucleotide biosynthetic process	5.9E-05	Disaccharide metabolic process	2.2E-04
N-acetylglucosamine metabolic process	1.0E-02	Citrate lyase complex	4.6E-03
Carbohydrate derivative metabolic process	1.6E-02	Lactose catabolic process via tagatose-6-phosphate	5.3E-03
Pathway name	p-Value	Pathway name	p-Value
Purine nucleotide biosynthesis	1.8E-06	Lactose and galactose degradation I	3.5E-04
Lactose degradation III	1.9E-02	Citrate degradation	4.7E-04
c) <i>Lb. helveticus</i>			
GO term	p-Value	GO term	p-Value
IMP dehydrogenase activity	2.0E-02	Fatty acid synthase activity	8.4E-04
Polyamine-transporting ATPase activity	2.0E-02	Beta-galactosidase activity	9.1E-03
Pathway name	p-Value	Pathway name	p-Value
Guanosine ribonucleotides de novo biosynthesis	2.0E-02	Fatty acid and lipid biosynthesis	4.4E-04
d) <i>P. freudenreichii</i>			
GO term	p-Value	GO term	p-Value
Carbohydrate-transporting ATPase activity	1.0E-02	NADH dehydrogenase activity	2.5E-06
Arginine metabolic process	1.3E-02	Electron transport chain	2.2E-05
Pathway name	p-Value	Pathway name	p-Value
Other amino acid biosynthesis	1.5E-03	Generation of precursor metabolites and energy	3.6E-09
Pyrimidine nucleotides salvage	4.0E-02	Fermentation	1.3E-06

hypothetical protein genes. In addition, some strain specific genes for *Lc. lactis*, such as citrate lyase genes, were also part of those missing regions. We also saw PTS system transporter genes and metal ion transporter genes for *Lb. helveticus*. Limitations of metagenomics assembly could be also reason for the missing regions.

On the data classification step, we detected bacteriophage marker genes in metagenomic DNA reads (Fig. S1 and S2 in Supplemental file 1). Phages, especially lactococcal phages, are commonly seen in cheese production and can have negative effects on fermentation (Deveau et al., 2006). The phages found in the studied cheese could have emerged from several sources. It has been reported that several phages can survive pasteurization and it is common to see airborne phages in cheese manufacturing plants (Verreault et al., 2011). Although these phages can affect negatively to cheese production, the cheese was successfully produced in this study. We analyzed several phage genes by using the metatranscriptomic data and saw several expressed phage genes included phage regulatory protein Rha, phage lysin, phage protein restriction nuclease, phage transcriptional regulator Cro/C1 family, phage antirepressor protein, and phage envelope protein. This suggests

that the phages were active during cheese ripening. Another cheese study (Chapot-Chartier et al., 1994) reported that two *Lc. lactis* sub-species behave differently during ripening process. Cell viability of *Lc. lactis* subsp. *cremoris* rapidly decreases due to lysis during the first week of ripening, while cell viability of *Lc. lactis* subsp. *lactis* decreases very slowly even at the last stages of cheese ripening (Chapot-Chartier et al., 1994). Furthermore, French cheese metagenomic study (Ceugniet et al., 2017) also showed the dramatical population decrease of *Lc. lactis* subsp. *cremoris* at the beginning of cheese ripening. This may explain why read coverage of *Lc. lactis* subsp. *cremoris* was very low (Table S1 and S2 in Supplemental file 1) and we were not able to reconstruct two different *Lc. lactis* genome.

Based on the mean DNA-seq and RNA-seq read coverage data, *Lc. lactis* strongly dominated the Maasdam cheese microbiota during the whole ripening process. Ojala et al. (2017) also showed that most of the amplicon sequencing reads were assigned to *Lactococcus*, while *Propionibacterium* and *Lactobacillus* were the next most abundant bacterial genera in same cheese samples. Earlier studies (Kocaoglu-Vurma et al., 2008; O'Sullivan et al., 2015, 2016), which used *S. thermophilus* and *Lb. helveticus* as starter cultures for another Swiss-type cheese, showed that both starter cultures dominated the cheese microbiota during cheese ripening. A microbiome study of Italian cheeses showed that *Lb. helveticus* is one of the major species in cheese microbiota in the presence of *Lc. lactis* and *S. thermophilus* (De Filippis et al., 2014). However, in our study *Lb. helveticus* had dramatically lower DNA and RNA read coverage compared to *Lc. lactis*. In addition, we observed that 75% of *Lb. helveticus* genes were low expressed during cheese ripening. There is evidence that sequencing depth affects the number of active genes and approximately two million of coding sequence-mapped reads provides saturation for the number of active genes (Landstorfer et al., 2014). In our study, even though the high number of total RNA-seq reads was obtained from cheese samples, only ~1,500,000 reads were mapped to annotated genes of *Lb. helveticus* at each timepoint. This may partially explain the high number of low expressed genes on *Lb. helveticus*. Despite the limited number of expressed genes, expression of proteinase and peptidase genes in *Lb. helveticus* were detected. These *Lb. helveticus* genes are some of the most important genes for cheese ripening acceleration. Mean coverage data also showed that the amount of *P. freudenreichii* increased significantly during warm room ripening.

The annotation results suggest that all four bacterial species play an important role in Maasdam cheese flavor development by proteolysis and peptidolysis. Proteolysis of casein plays a critical role in cheese ripening since it provides small peptides and free amino acids (Savijoki et al., 2006), which directly affect flavor. We showed that all three lactic acid bacteria strains carried proteinase genes for casein degradation. Even though *P. freudenreichii* subsp. *shermanii* JS had some proteinase genes such as Clp protease genes and serine protease HtrA gene, a casein degradation related proteinase gene was not found. Low proteinase activity of propionibacteria has also been reported in earlier studies (Baer, 1995). In addition, peptide transporter genes and peptidase genes were found in all four strains. These genes indicate that not only lactic acid bacteria but also *P. freudenreichii* subsp. *shermanii* JS has potential to use peptides that are derived from casein degradation.

Earlier studies (Feeney et al., 2001; Pachlová et al., 2012) reported that elevated temperature accelerates the ripening process due to increased proteolytic and peptidolytic activities. Our data showed that in the cold room, none of the casein related proteinase genes were downregulated. *Lb. helveticus* PepX and *Lb. rhamnosus* PepV peptidase genes were downregulated during cold room ripening, but other peptidase genes were not. Interestingly, PepQ peptidase gene of *Lc. lactis* was upregulated during cold room ripening. Therefore, for Maasdam cheese, we believe that transition from the warm to the cold room did not affect significantly the expression levels of the protease and peptidase related genes.

Degradation of released free amino acids is a key factor for the formation of several flavor compounds in cheese such as aldehydes,

alcohols, carboxylic acids and sulfur compounds. Branched-chain amino acid degradation by cheese microbiota leads to formation of malty and fruity flavors to cheese (Ardö, 2006). Valine degradation pathways were found for *Lc. lactis* and *P. freudenreichii*. In addition, degradation of sulfur containing amino acids provides the key volatile sulfuric compounds (Seefeldt and Weimer, 2000), such as methanethiol and hydrogen sulfide. Our annotation results showed that *Lc. lactis*, *Lb. rhamnosus*, and *Lb. helveticus* had genes for the degradation of sulfuric amino acids. Furthermore, likewise the expression of proteolytic and peptidolytic related genes, transition from the warm to the cold room did not affect significantly expression of aminotransferase genes.

Lactic acid bacteria and propionibacteria are known as vitamin producers (Leblanc et al., 2011). Our study showed that Maasdam cheese microbiota carried genes for production of several vitamins, such as vitamin B₁, B₆, and B₁₂, it potentially increases the nutritional value of cheese. In addition, gene expression analysis showed that *Lc. lactis* genes *ribBA*, *ribF*, *tenA*, and *thiD* for vitamin B₁ and B₂ production were significantly upregulated after transition to the cold room.

During cheese making the starters ferment lactose to pyruvate, which is further converted to several flavor compounds (Fig. 6). In cheese, the main source of diacetyl and R-acetoin, which provide buttery flavor, is the citrate catabolism (Martino et al., 2016). Unlike lactose, few lactic acid bacteria can utilize citrate (Smid and Kleerebezem, 2014). In the studied Maasdam cheese only *Lb. rhamnosus* possesses all necessary genes for citrate degradation. Citrate catabolism produces CO₂, therefore playing a role in eye formation in Dutch-type cheese (O'Sullivan et al., 2016). Genome annotation of *P. freudenreichii* showed potential responsible genes for propionate production from pyruvate and the TCA cycle, which also provides CO₂.

From the same cheese sampling (Ojala et al., 2017), it was shown that the central metabolism of *P. freudenreichii* is less active during cold room ripening. As expected, our results overlapped with results of earlier study (Ojala et al., 2017). Several pathways of *P. freudenreichii* were downregulated during cold room ripening. Downregulation of TCA cycle and aerobic respiration genes suggests that in cold room ripening *P. freudenreichii* growth was slower. In general, genes responsible for fermentation of pyruvate were significantly downregulated in the cold room, which suggests potentially lower production of propionate, diacetyl, R-acetoin, and acetate. In *P. freudenreichii* pyruvate is produced mostly from lactate. We suggest that concentration of lactate in the environment should have increased due to the fermentative metabolism of lactic acid bacteria. Therefore, we suppose that source limitation could not be the reason of decreased pyruvate utilization. We suggest that pyruvate utilization genes were downregulated during cold room ripening because growth and metabolism of *Propionibacterium* slows down in cold room temperature.

Similar to *P. freudenreichii*, we observed that central metabolism genes of *Lb. helveticus* and *Lb. rhamnosus* were downregulated. Downregulation of lactose utilization and citrate degradation genes in *Lb. rhamnosus* in the cold room suggests potentially decreased production of pyruvate and buttery flavor in cold room ripening. Another Swiss-type cheese study also reported that citrate level decreases significantly when cheese enters the warm room (O'Sullivan et al., 2016). Downregulation of genes that are related to important cofactor compound production pathways also supports the hypothesis that energy metabolism of *Lb. rhamnosus* was less active in the cold room possibly due to the shortage of lactose and citrate. Cold room ripening caused downregulation of fatty acid production genes in *Lb. helveticus*. Palmitic acid and stearic acid have bitterness-masking effects on cheese (Homma et al., 2012), therefore downregulation of fatty acid production genes in *Lb. helveticus* may affect cheese flavor. Another metatranscriptomic study (De Filippis et al., 2016) on cheese also showed that fatty acid biosynthesis genes are more highly expressed during the high temperature ripening.

Interestingly, unlike other species, metabolic genes of *Lc. lactis* were not downregulated during cold room ripening. Instead of

downregulation, we observed upregulation of few central metabolism pathways including homolactic fermentation and vitamin production pathways. In the homolactic fermentation pathway, significantly up-regulated L-lactate dehydrogenase genes (*ldh*) suggest that in the cold room more pyruvate could be converted to L-lactate compared to the warm room. However, another study (Ong and Shah, 2008), which used *Lc. lactis* as a cheddar cheese starter, showed that concentration of lactic acid was significantly higher at 8 °C and 12 °C compared to 4 °C ripening. We did not observe other pyruvate converting enzymes changing their expression significantly. Our observation of upregulation of metabolic processes in cold room ripening may be explained by decrease of carbon source availability. It was reported that, on the late stage of ripening, during energy starvation conditions, the expression of *ldh* gene of *Lc. lactis* is enhanced (Bachmann et al., 2010). In mixed culture, over-expression of *ldh* gene of *Lc. lactis* provides survival advantage over other species during cheesemaking (Desfossés-Foucault et al., 2014). Furthermore, it was reported that during carbon starvation, *Lc. lactis* remains metabolically active and reach nonculturable state (Ganesan et al., 2007).

During cold room ripening, we observed that *Lc. lactis* genes *uxuB*, *kdgA*, and *mtlD* for fructuronate and mannitol degradation were upregulated. In addition, *Lb. rhamnosus* genes *nagA* and *nagB* for N-acetylglucosamine degradation also were upregulated. Upregulated degradation of sugar derivatives' genes may indicate that in the cold room, the main carbohydrate, lactose, and citrate, were depleted. Another study (O'Sullivan et al., 2016) also reported that during Swiss-type cheese ripening after day 35 the absence of lactose and galactose is observed. In addition, Emmental Swiss-type study showed that citrate is depleted after the fourth week of cheese ripening (Weinrichter et al., 2004). Therefore, it is possible that in addition to the temperature change, there could be other factors, such as carbohydrate depletion and metabolic competition between starter cultures (Desfossés-Foucault et al., 2014; Weinrichter et al., 2004), that explain the gene expression changes found between the warm and cold room ripening.

In the cold room, we observed upregulated acid stress resistance related genes in cheese microbiota. In addition, upregulated polyamine transporter gene (*potA*) in *Lb. helveticus* can be associated with acid resistance due to positive impact of putrescine on acid stress survival (Romano et al., 2014). Most significantly upregulated genes in the cold room for *Lb. rhamnosus* and *Lb. helveticus* were purine and guanosine nucleotide biosynthesis genes, respectively. Purine metabolism genes and guanine nucleotides have a role in stress resistance (Rallu et al., 2000; Xie et al., 2004). Upregulated acid resistance genes during cold room ripening may indicate the increase of acidity in cold room ripening.

5. Conclusion

Metagenomic and metatranscriptomic analysis of the Maasdam cheese gave us an opportunity to identify microbiota of the cheese and determine gene responses during ripening process.

We reconstructed four genomes from cheese metagenome data with high level of completeness; *Lactococcus lactis*, *Lactobacillus rhamnosus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii* subsp. *shermanii* strain JS. Based on mean coverage, *Lc. lactis* dramatically dominated in the cheese microbiota. Annotation and pathway analysis of these four genomes showed that all four species are potentially important for cheese flavor development and the responsible gene activities were observed from the RNA-seq data.

Gene responses during warm and cold ripening suggest that carbon sources like lactose and citrate possibly decreased by day 37. Cold room temperature and limited carbon source availability caused downregulation of genes that are responsible for flavor compound production in *Lb. rhamnosus* and *Lb. helveticus*. Despite decreased availability of carbon source and cold room temperature, *Lc. lactis* was metabolically active and genes for homolactic fermentation and vitamin B₁ and B₂

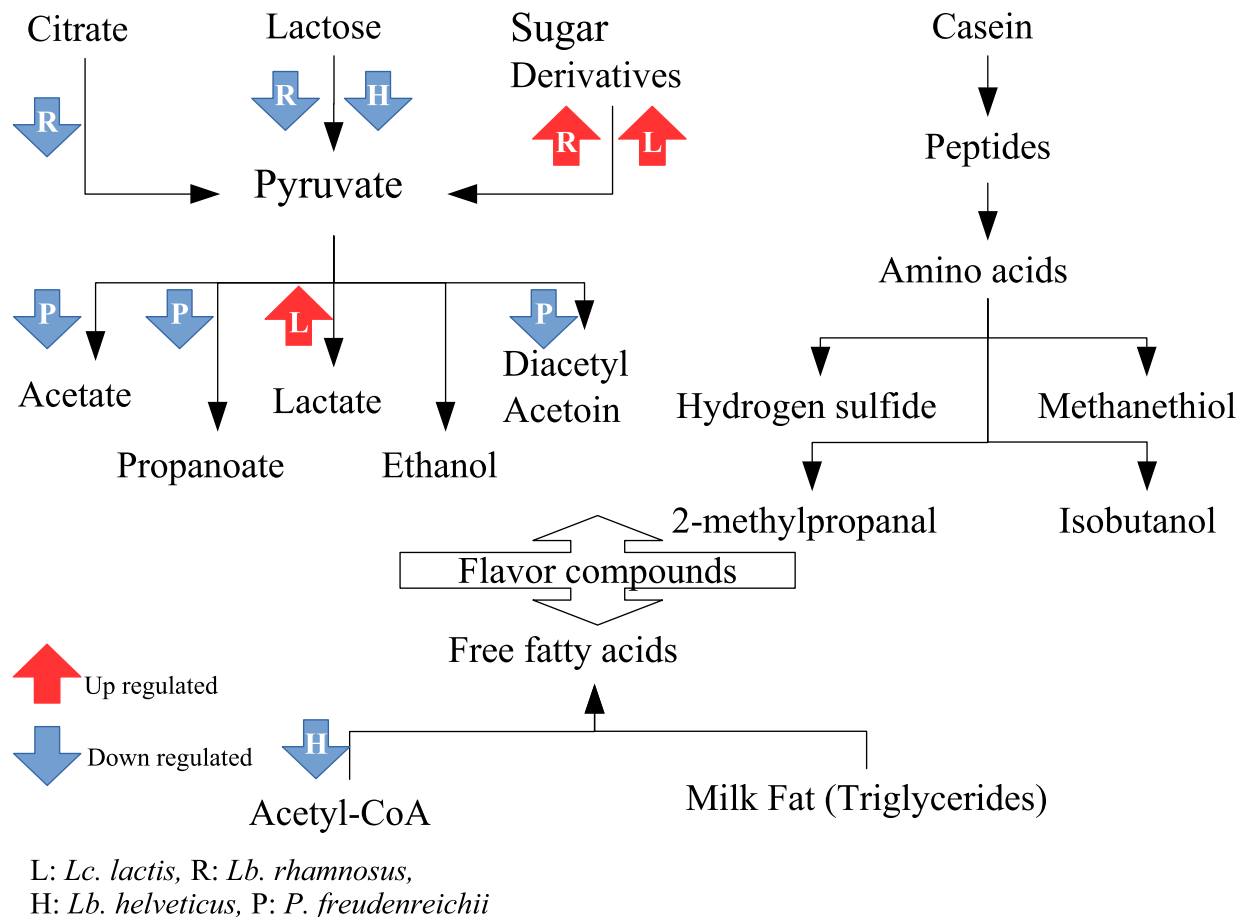


Fig. 7. Summary of flavor compound producing processes in Maasdam cheese. Upside red arrows represent that pathway was upregulated during cold room ripening. Downside blue arrows represent that pathway was downregulated during cold room ripening. Letters represent species; R: *Lb. rhamnosus*, L: *Lc. lactis*, P: *P. freudenreichii*, H: *Lb. helveticus*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

synthesis were upregulated during cold room ripening. We believe that in Maasdam cheese, the decrease in carbon source first affected other species than *Lc. lactis*. Critical energy production and central metabolism genes of *P. freudenreichii* subsp. *shermanii* strain JS were down-regulated during cold room ripening despite the simultaneous higher expression of lactate production genes in *Lc. lactis*. Overall, flavor related genes in cheese microbiota were downregulated during cold room ripening except in *Lc. lactis* (Fig. 7).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2018.05.017>.

Data availability

All sequencing data and contigs have been deposited in the European Nucleotide Archive (ENA) under accession code PRJEB23938.

Conflict of interest

SK and ST are employed by Valio Ltd.

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